Expanded View Figures

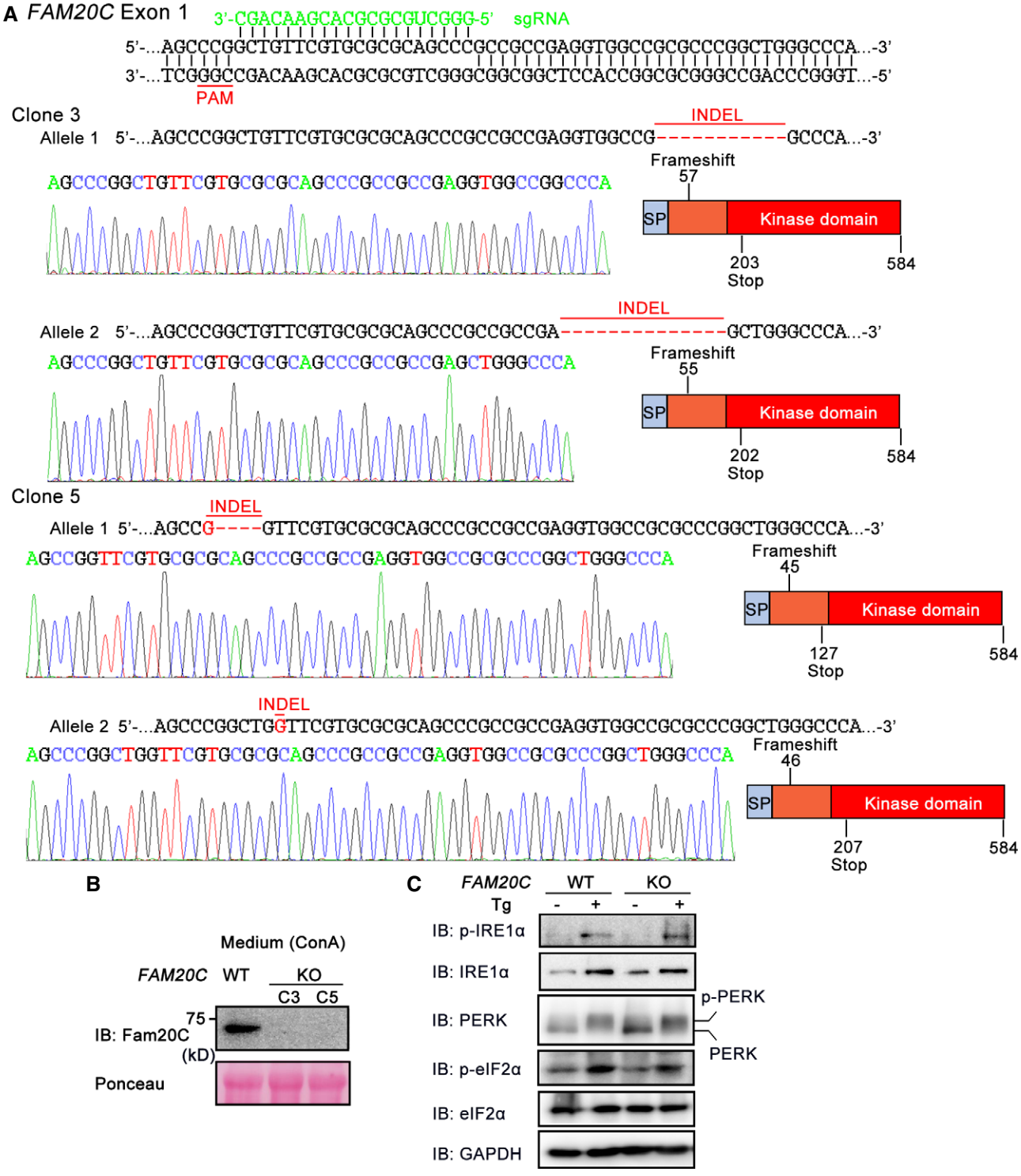

Figure EV1.

Figure EV1. Generation of *FAM20C* KO HeLa cells using CRISPR/Cas9 genome editing.

A Schematic representation of the base pairing between the guide RNA (sgRNA) and the targeting locus of exon 1 in the human *FAM20C* gene. The sequences of the mutated alleles in *FAM20C* clone 3 (C3) and clone 5 (C5) and representative chromatograms depicting the insertions/deletions (INDELs) are shown. The INDELs were predicted to cause frameshift mutations producing inactive copies of the protein. SP, signal peptide.

B Protein immunoblotting of Concanavalin A-Sepharose (ConA) precipitates from the culture medium of WT and *FAM20C* KO HeLa cells. Ponceau staining was used as a loading control.

C *FAM20C* KO shows little effect on unfolded protein response signaling. Protein immunoblotting of cell extracts from WT and *FAM20C* KO HeLa cells treated with or without 5 μM Tg for 6 h.

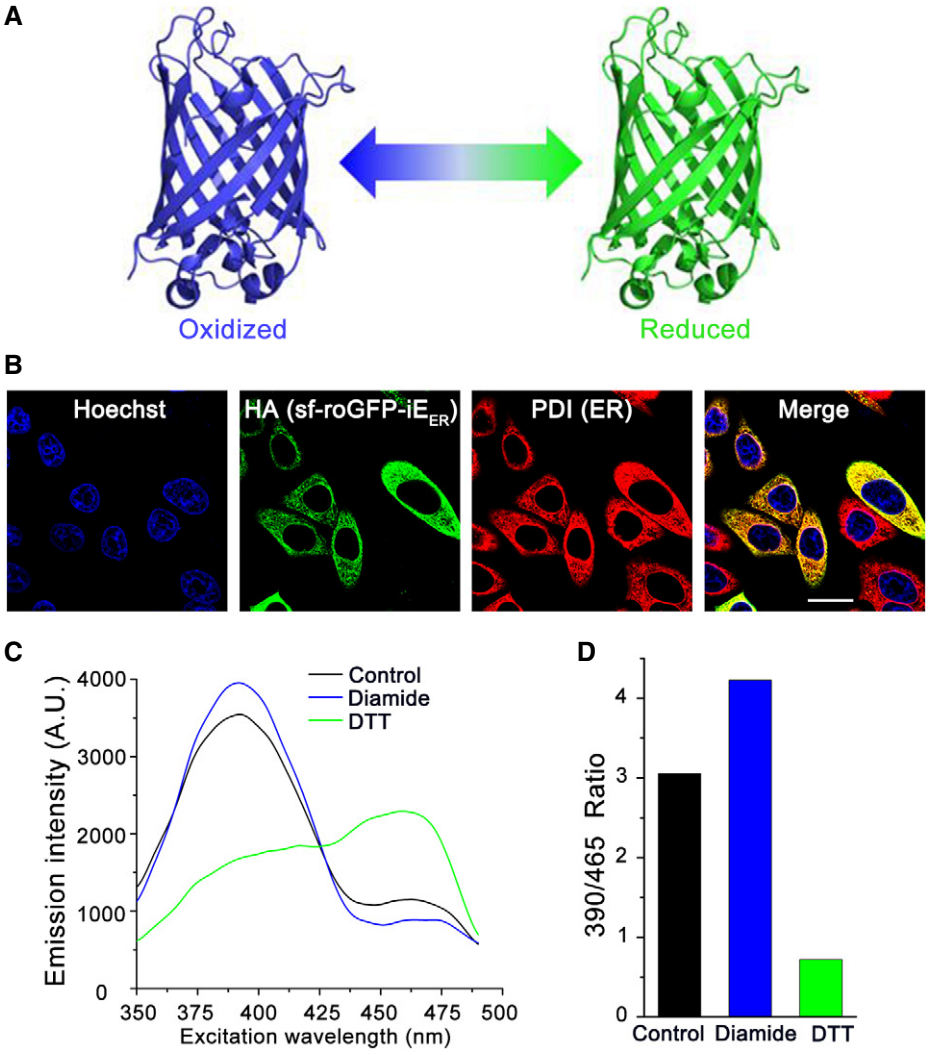

Figure EV2. Redox response ability of the superfolded-roGFP-iE$_{ER}$ in HeLa cells.

A Schematic representation of superfolded-roGFP-iE$_{ER}$ exhibiting different fluorescence characteristics under oxidizing (blue) or reducing (green) condition.

B Localization of superfolded-roGFP-iE$_{ER}$ in HeLa cells analyzed by immunofluorescence. Scale bar = 20 μm.

C Fluorescence excitation spectrum of superfolded-roGFP-iE$_{ER}$ in HeLa cells untreated or treated with 1 mM diamide or 1 mM DTT.

D The ratios of fluorescence intensities at 525 nm with excitation at 390 and 465 nm were calculated from (C).

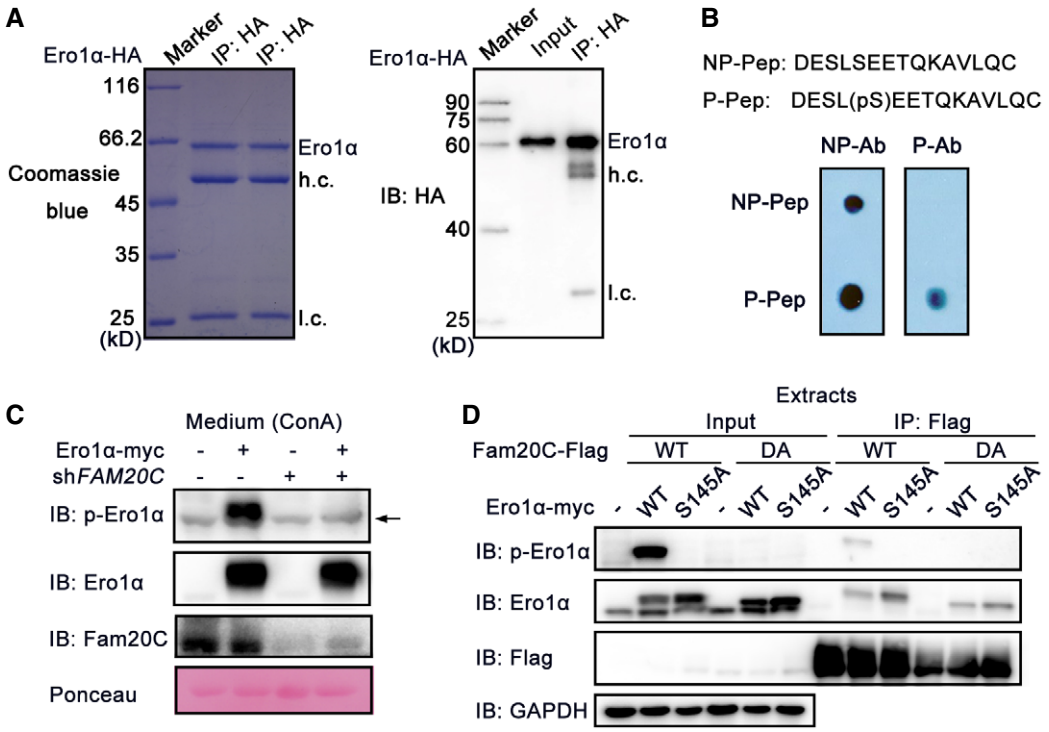

Figure EV3. Fam20C is the kinase catalyzing Ero1α phosphorylation.

A Coomassie Blue Staining (*Left*) and protein immunoblotting (*Right*) of HA immunoprecipitates from HeLa cells expressing Ero1α-HA. h.c.: heavy chain, l.c.: light chain.

B Non-phosphopeptide (NP-Pep) and phosphopeptide (P-Pep) used for generating non-phospho-antibody (NP-Ab) and phospho-antibody (P-Ab). 5 ng NP-Pep and P-Pep were spotted on membranes separately, followed by dot blot analysis with NP-Ab and P-Ab, respectively.

C Protein immunoblotting of ConA precipitates from the conditioned medium of HeLa cells transfected with Ero1α-myc and/or shRNA targeting *FAM20C*. The arrow indicates an unspecific background band.

D Co-immunoprecipitation of Ero1α and Fam20C in HeLa cells expressing Fam20C-Flag WT/DA and/or Ero1α-myc WT/S145A.

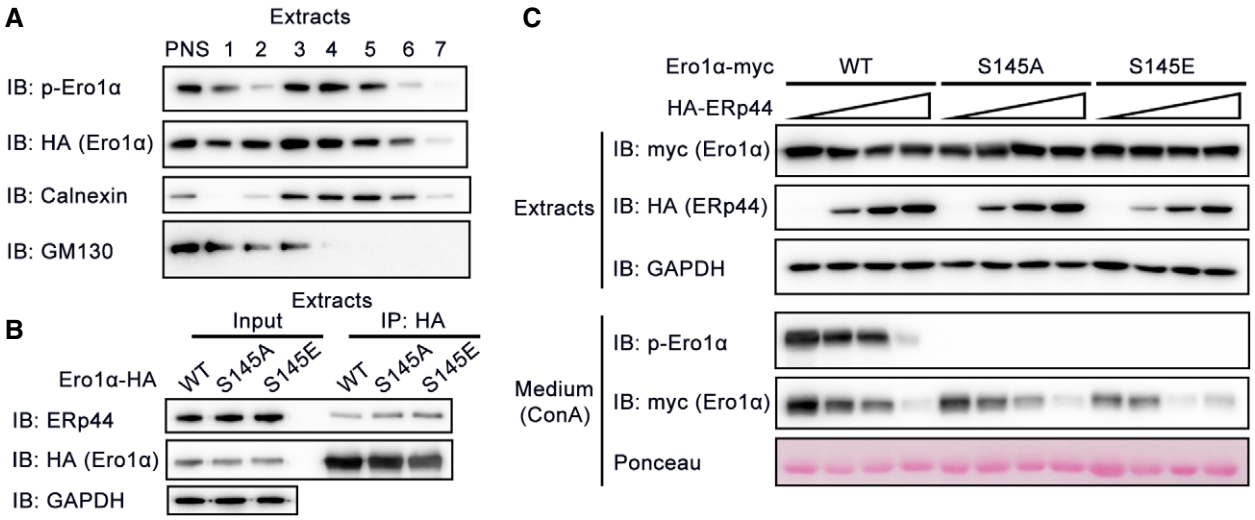

Figure EV4. The ER retention of p-Ero1α is mediated by ERp44.

A Subcellular fractionation of HeLa cells. The postnuclear supernatant (PNS) of HeLa cells expressing Ero1α-HA and Fam20C-Flag was separated on a 30% Percoll gradient and the fractions were collected and subjected to protein immunoblotting. Calnexin, ER marker; GM130, Golgi marker.

B Co-immunoprecipitation of Ero1α and endogenous ERp44 in HeLa cells expressing Ero1α-HA WT/S145A/S145E.

C Protein immunoblotting of cell extracts and ConA precipitates from the conditioned medium of HeLa cells overexpressing Ero1α-myc WT/S145A/S145E and increasing amounts of HA-ERp44.

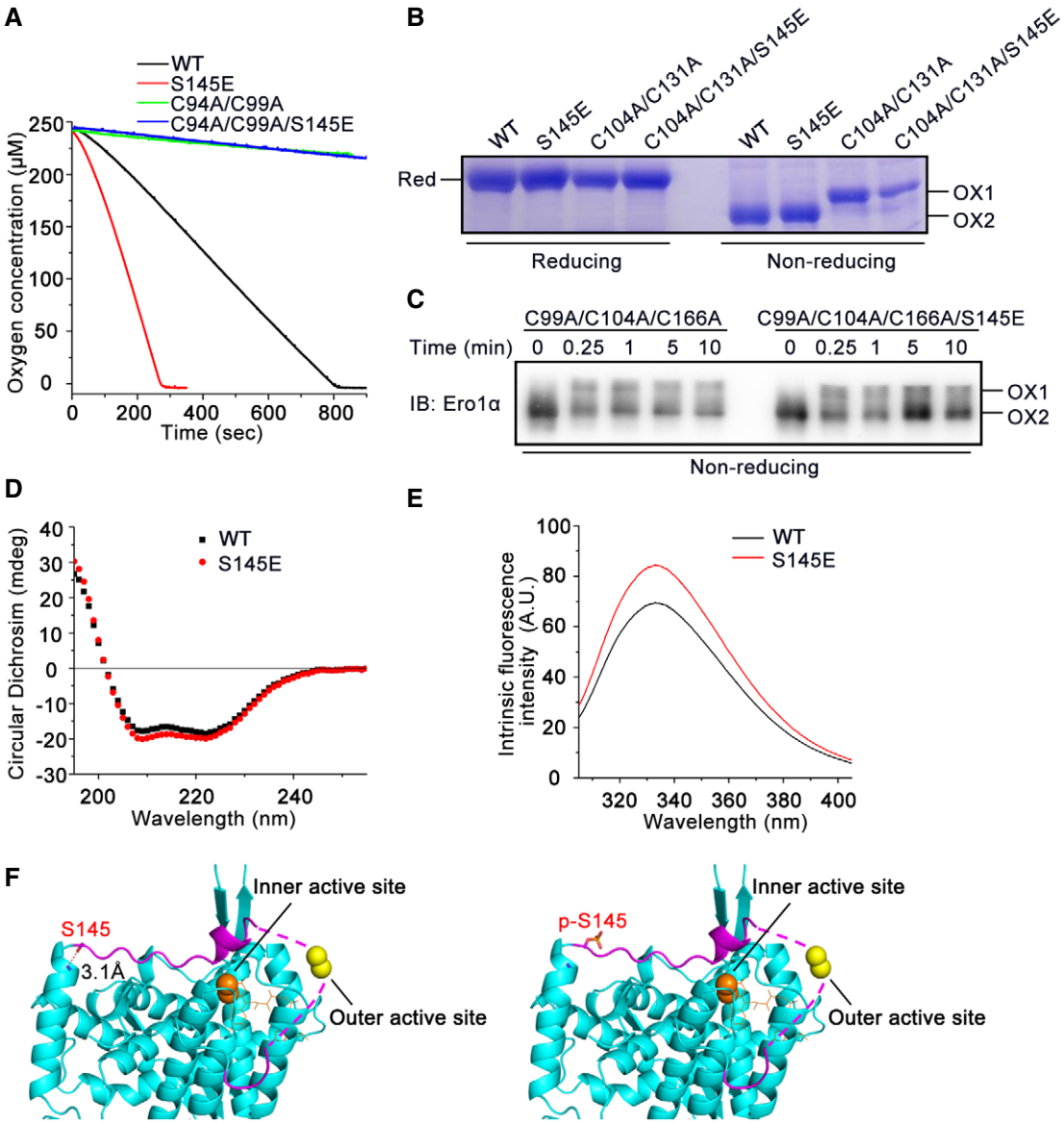

Figure EV5. Biophysical and biochemical characterization of Ero1α phosphorylation mimics.

A Oxygen consumption catalyzed by Ero1α WT, inactive Ero1α C94A/C99A, and corresponding phosphorylation mimics was monitored in the presence of PDI and GSH.

B The redox states of recombinant Ero1α WT, hyperactive Ero1α C104A/C131A, and corresponding phosphorylation mimics were determined under reducing and non-reducing conditions. The active form (OX1), inactive form (OX2), and fully reduced form (Red) are indicated.

C Recombinant oxidized Ero1α C99A/C104A/C166A and Ero1α C99A/C104A/C166A/S145E were mixed with reduced PDI, and aliquots were taken at indicated times for analysis by non-reducing SDS–PAGE and Ero1α blotting.

D Far UV circular dichroism spectrum of recombinant Ero1α WT and S145E.

E Intrinsic fluorescence spectrum of recombinant Ero1α WT and S145E.

F Ribbon representation of non-phosphorylated Ero1α (*Left*) and Ser145-phosphorylated Ero1α (*Right*) based on the structure of active human Ero1α (PDB: 3AHQ). The outer active site-containing loop is shown in lilac, and the missing region in dashed line. Outer active site (yellow ball), inner active site (orange ball), cofactor FAD (orange stick), and the side chains of Ser145 and p-Ser145 (stick) are indicated. The distance between *O* atom of Ser145 side chain and *N* atom of Thr148 main chain is shown.

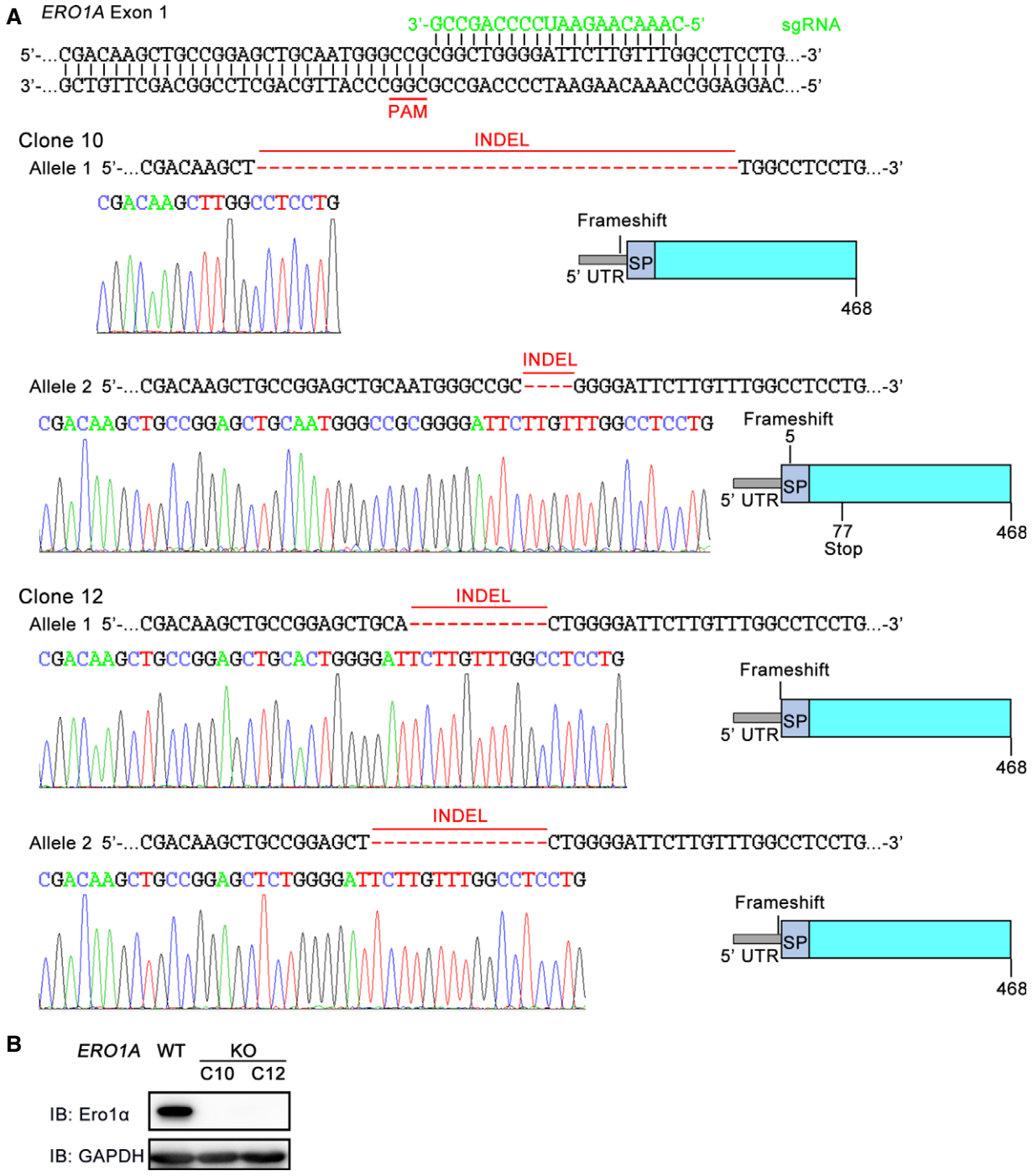

Figure EV6. Generation of *ERO1A* KO HeLa cells using CRISPR/Cas9 genome editing.

A Schematic representation of the base pairing between the sgRNA and the targeting locus of exon 1 in the human *ERO1A* gene. The sequences of the mutated alleles in *ERO1A* clone 10 (C10) and clone 12 (C12) and representative chromatograms depicting the INDELs are shown. The INDELs were predicted to cause frameshift mutations producing inactive copies or terminating translation of the protein. SP, signal peptide.

B Protein immunoblotting of cell extracts from WT and *ERO1A* KO HeLa cells.